iPS cell therapy for Parkinson’s disease
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CHAPTER 5

PARTICIPATION OF PERFORIN IN MEDIATING DOPAMINERGIC NEURON LOSS IN MPTP-INDUCED PARKINSON’S DISEASE IN MICE

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ABSTRACT

Parkinson's disease (PD) is gradually recognized as a neuroinflammatory disease. Both resident innate and peripheral immune aberrations have been observed in PD patients and they have been demonstrated to influence disease progression in animal models. However, it is still enigmatic how and which immune components are lethal to the dopaminergic neuron in PD. Here we showed that perforin was significantly increased in the serum of wild-type mice 4 weeks after an i.p. injection of 1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin used to induce PD-like symptoms in mice. We made use of perforin-knockout mice and demonstrated that perforin-deficiency attenuated the acute striatal dopamine reduction in the MPTP-treated mice by 33% and abated microglia activation 3 days post MPTP-injection. Perforin-deficiency appeared to retard dopaminergic neuron death in the MPTP-mice resulting in striatal dopamine recovery twice as high as for wild-type mice 4 weeks post MPTP-injection. Our study suggests that perforin plays a role in dopaminergic neuron loss in PD.
INTRODUCTION

After many decades of research, the mechanisms underlying the pathogenesis of Parkinson's disease (PD) are still not fully understood. Among the many cellular mechanisms proposed, the contribution of neuroinflammation in PD pathogenesis has gained more and more attention. Microglia activation, as resident innate immune reaction, has been well demonstrated in PD [1].

The changes in peripheral lymphocyte constitution are significant in PD patients and animal models [2]. Including an increase in γδT cells, CD45RO+ memory T cells [3] and NK cells [4] as well as a decrease in the total count of T cells and B cells [5-6]. Infiltration of CD4+ T cells and CD8+ T cells in the brain parenchyma has been described [7-9]; in particular, CD8+ T cells appear to be prominent with their number 4.8 times as high as CD4+ T cells [9]. These results clearly indicate the involvement of these cells in PD development.

Recent studies revealed several molecular mechanisms, in which these immune cells contribute to dopaminergic (DA) neuron loss. It was proposed that IFN-γ is critical in microglia-mediated loss of DA neurons in 1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice [10]. The deleterious activity of infiltrated CD4+ T cells seems to involve the Fas/FasL pathway [9]. Although not present in the brain parenchyma, B cells may contribute to DA neuron loss by secreting IgG that binds to the Fcγ receptor and modulates the microglial response [11]. Another potential factor involved in DA neuron injury could be perforin (Prf1). Perforin is a pore-forming protein and is employed mostly by cytotoxic CD8+ T and NK cells to eliminate target cells via the delivery of granzymes [12]; also murine CD4+ Th1 cells seem to express Prf1 mRNA [13]. It has been shown that perforin from NK cells, CD8+ T cells and CD4+ Th cells can mediate neurite damage [15-16] or neuron death [17] in vitro and it has been suggested to participate in neuronal degeneration in multiple sclerosis [18].

In this study, we set out to obtain first evidence for the potential involvement of perforin in dopaminergic degeneration. We applied MPTP to induce DA neuron degeneration in perforin-deficient mice (Pfp−/−) as well as in wild-type (WT) control mice. After assessing the increase in the perforin serum level due to MPTP, we have monitored the striatal dopamine level and the actual dopamine neuron loss during 4 weeks in both groups. In addition, we compared the changes in MPTP-induced microglia activation in the perforin-deficient mice (Pfp−/−) as well as in the wild-type (WT) control mice.

MATERIAL AND METHODS

Animals

Eight- to twelve-week-old male mice, weighing 22–26 g were used. The following strains were obtained from Taconic, Borup, Denmark: Pfp−/− (B6.129S6- Prf1tm1Clrk N12) and corresponding WT inbred C57BL/6]. The animals were maintained under standard specific pathogen-free conditions and allowed access to food and water ad libitum. All experimental
procedures were approved by the Animal Ethics Committee of Shantou University Medical College (People’s Republic of China).

**Treatment and tissue preparation**

Groups of mice received 4 times of i.p. injections of MPTP-HCl (15 mg/kg, at 2-hour intervals) and were sacrificed from 3 to 28 days after the last injection; control mice received PBS only. Blood samples were drawn from the heart after isoflurane anesthesia. For obtaining fresh striatum, mice were transcardially perfused with PBS only. The striata were quickly dissected, frozen on dry ice and stored in -80°C. For immunohistochemistry, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3. Tissues were postfixed in 4% PFA at 4°C overnight, kept in 20% sucrose at 4°C for 1 day, embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, USA), and snap frozen in 2-methyl-butane (isopentane) precooled to –70°C. Twenty-five micrometer thick cross-sections were cut using a cryostat (CM1850, Leica, Nussloch, Germany). Series of sections with 10-sections-intervals were collected.

**Measurement of striatal dopamine**

Dopamine Research ELISA kit (Dopamine Research RIA, Labor Diagnostika Nord Gmbh & Co. KG, Germany) was used to measure the dopamine level in striatum. On the day of analysis, striata were homogenized in 0.01 N HCl with 1mM EDTA and 4 mM sodium metabisulfite. Protein concentrations were measured using BCA protein assay (Pierce). Samples were processed according to the manufacturer’s instructions while ensuring proper standards and controls.

**Immunohistochemistry**

After antigen retrieval, sections were incubated in PBS containing 0.2% v/v Triton X-100, 0.02% w/v sodium azide and 5% v/v normal serum (according to secondary antibody used) for 1 hr. Primary antibodies (anti-TH 1:800, Millipore; anti-Iba1 1:800 Millipore) were then applied for 24 hrs at 4°C. For detection of the primary antibodies, appropriate secondary antibodies, coupled to Cy2 or Cy3 (1:800, Jackson ImmunoResearch, West Grove, USA) were used. Sections were counterstained for 10 minutes with 50 μg/ml DAPI (Sigma) to visualize cell nuclei. Specimens were examined with an epifluorescence microscope (Axio Imager Z1, Zeiss, Oberkochen, Germany).

**Image and TH+ cell counting**

Fluorescent sections were analysed on a Zeiss Axiolmager Z1. For each animal, one series of sections was stained with TH and DAPI. The TH+ neurons were counted in the right and left substantia nigra (SN) of every tenth section throughout the entire extent of the SN. An epifluorescence microscope (Axio Image Z1, Zeiss, Oberkochen, Germany) that was equipped with a motorized stage and a Stereo Investigator software-controlled computer system (MicroBright-Field, Magdeburg, Germany) was used for quantitative analysis. The border of SN and ventral tegmental area (VTA) was delineated at lower magnification based on TH
immunostaining. Using random sampling in the SN, cell counts were performed according to the optical dissector principle at a magnification of 400×.

**Cell counting of microglia**

Imaging was performed with a Zeiss AxioImager Z1 microscope. Sections of comparative levels in one section series were selected. Six images of Iba1 staining were taken from both the left and right sides of the dorsal striatum at 200× magnification. The number of Iba1+ cells in each image was counted, and data were presented as number of cells per image (450 µm × 337 µm).

**Analysis of perforin level**

Blood samples were drawn and kept at room temperature for 30 min before centrifugation at 3,000 rpm for 10 min. Serum was taken and stored at −70°C until further analysis. The concentration of perforin in serum was analysed with mouse perforin 1 (PRF1) Elisa kit (MyBioSource, Inc. San Diego, California, USA); samples were processed according to the manufacturer’s instructions.

**Data acquisition and statistical analysis**

All tests were performed and analysed in a blinded manner. Throughout the text and in the figures, all values are expressed as the mean ± SEM (standard error of the mean). Difference in means between 2 groups of independent samples was analysed using student’s t test. Differences in means among multiple data sets were analysed using one-way ANOVA by Dunnett’s post-hoc analysis. In all analyses, P values of less than 0.05 were considered significant.

**RESULTS**

**Perforin level is up-regulated in MPTP-lesioned wild type mice**

We first analysed the perforin level in serum from MPTP-intoxicated WT mice and compared it with the serum level in PBS-treated mice (Figure 1). A basal level of perforin was detected in the PBS group as well as in the MPTP-treated groups at the time point of 3 days, 1 and 2 weeks post injection. A significant increase of perforin was detected 4 weeks post MPTP intoxication. Since perforin is predominantly expressed by activated cytotoxic lymphocytes (and not by naive ones), our data suggest that MPTP induce activation of cytotoxic lymphocytes leading to the production of perforin and its release in the serum.

**Perforin-deficiency abated the MPTP-induced striatal dopamine level reduction**

To test the potential involvement of perforin in MPTP-induced PD development, perforin knockout (Pfp−/−) mice were used. Pfp−/− and WT mice were either intoxicated with MPTP or received PBS via intraperitoneal injections. The striatal dopamine levels of each group were determined at different time points (i.e. 3 days, 1, 2, 4 weeks) post injection. The dopamine levels in the striatum of the MPTP-treated groups were normalized to that of the related PBS-treated group (Figure 2.). As expected, the striatal dopamine level dramatically decreased in
both the Pfp⁻/⁻ and WT mice 3 days post MPTP injection. In the MPTP mouse model, DA neurons are initially distressed by the neural toxin MPP⁺, derived from the injected MPTP [24]. The acute drop in striatal dopamine level 3 days post MPTP injection may be ascribed to this first stress respond of DA neurons and not yet to actual neuronal loss. The significantly higher dopamine level in Pfp⁻/⁻ mice (41%) at 3 days in comparison to the WT mice indicates the involvement of perforin in early distress to DA neurons.

**Figure 1. Increased concentration of perforin in the serum of MPTP-intoxicated WT mice**
The concentration of perforin in serum of wild-type mice was significantly increased 4 weeks after MPTP treatment in comparison to PBS-treated control. * P < 0.05 (one way ANOVA, Dunnett t-test. n = 4-6 in each group).

The dopamine levels in WT mice treated with MPTP changed over time: it decreased to the lowest at 3 days and increased slowly to around 40% by 4 weeks post injection. This recovery to about 40% presumably points to a stabilization of stress and the level of degeneration of DA neurons. In comparison to the WT mice, perforin-deficient mice showed a slower pattern and less reduction of the striatal dopamine level. The lowest dopamine level was found at 1-week post injection and was around 20% of PBS-treated Pfp⁻/⁻ mice. The dopamine level in the Pfp⁻/⁻ mice quickly increased to 80% at 4 weeks after MPTP injection. At this time point, the dopamine level in perforin-deficient mice was significantly higher than in wild type mice. These results suggest less stress and a better survival of SN DA neurons in perforin-deficient mice after MPTP intoxication.

**Figure 2. Reduction of striatal dopamine level in MPTP-intoxicated mice**
Striatal dopamine levels dramatically decreased 3 days post MPTP treatment and recovered over time, most prominently in the Pfp⁻/⁻ mice up to 4 weeks post MPTP treatment. Each MPTP-treated group is compared with its respective PBS control * P < 0.05, ** P < 0.001 (One way ANOVA, Dunnett’s post-hoc analysis. n = 6-8 in each group.)
**Figure 3.** Perforin-deficiency abates MPTP-induced SN DA neuron degeneration

(A) Representative staining of TH in the SN of wild type and Pfp−/− mice. Shown are PBS control and MPTP-treated mice sacrificed at 3 days, 1, 2 and 4 weeks post MPTP injection. Scale bar is 200 μm. (B) Survival of TH+ cells in the SN of MPTP-lesioned wild type and Pfp−/− mice. TH+ cells were quantified with unbiased Stereo Investigator software-controlled computer system. *P < 0.05 (Student’s t test; One way ANOVA, Dunnett t-test. n = 3 or 4 in each group).

**Perforin-deficiency reduced dopaminergic neuron degeneration in the SN of MPTP-intoxicated mice**

To examine the effect of perforin-deficiency on the survival of SN DA neurons, we analysed the dynamic changes of the number of DA neurons in SN of Pfp−/− and WT mice following MPTP intoxication (Figure 3). TH+ cells were counted by Stereo Investigator, and the border
between the SN and VTA was defined under the microscope. In WT mice, the number of DA neurons gradually decreased over time from 3 days up to 4 weeks post MPTP injection compared to PBS-treated wild-type mice; the difference between the MPTP group and the PBS control group was significant at 4 weeks post MPTP injection. However, the number of SN DA neurons in the Pfp⁻/⁻ mice treated with MPTP did not significantly change within the period of time studied. By week 4, the number of DA neurons in the MPTP-treated Pfp⁻/⁻ group was significantly higher than that in the MPTP-treated WT mice (Figure 3B). These results demonstrate the involvement of perforin-mediated DA neuron loss and the subsequent reduction in dopamine production in MPTP-treated mice.

**Figure 4. Microglia activation in the striatum of the MPTP-intoxicated mice**
Staining of Iba1 in the striatum of wild-type (A-E) and Pfp⁻/⁻ (F-J) mice. PBS-injected mice (A, F) were sacrificed at day 3 as control. Dynamic changes of Iba1⁺ cells are shown at 3 days (B, G), 1 week (C, H), 2 weeks (D, I) and 4 weeks (E, J) post MPTP injection. Microglia activation was detected 3 days after MPTP-injection evidenced by retracted pseudopodia and a larger cell body (b, g). The scale bar is 100μm in A-J, and is 10μm in a-j. (K) The dynamic changes in the number of microglia (number of cells/image area size (450 μm × 337 μm)). In WT mice, the number of microglia at MPTP 3D group is significantly different in comparison to all other groups; in Pfp⁻/⁻ mice, the number of microglia in the MPTP 3D group is significantly different to the other groups indicated. * P < 0.05 (Student’s t test; One way ANOVA, Dunnett’s post-hoc analysis. N = 3 in each group).

**Microglia activation in Pfp⁻/⁻ mice and wild type mice after MPTP treatment**
Microglia activation in the striatum and SN has been demonstrated in post-mortem brain tissue in PD patients as well as in animal models for PD [8, 25]. The activation of the microglia in the meso-striatal pathway is thought to be induced by the (stress) signals released by the endangered, degenerating SN DA neurons due to MPTP intoxication. We analysed the extent of activation of microglia in the striata of the Pfp⁻/⁻ and WT MPTP-treated mice based on the morphology (e.g. presence of retracted pseudopodia) and the number of microglia (detected
by Iba1 immunostaining) (Figure 4). A significant microglia activation was detected in both the Pfp+/− and (even higher) in the WT mice only at 3 days post MPTP intoxication (Figure 4K); this appeared to be consistent with previous findings that microglia are transiently activated in the acute phase of MPTP-lesioned mice [8]. The activation state of the microglia at day 3 was also reflected in the activated cell morphology with retracted pseudopodia (Figure 3b&g), and also in the number of Iba1+ microglia (Figure 4K). In WT mice, the number of Iba1+ cells was significantly higher at day 3 in comparison to Pfp−/− mice (Figure 4K).

**DISCUSSION**

Our experiments with MPTP toxification in perforin-knockout mice strongly suggest that perforin is involved in the injury and loss of dopaminergic neurons in this animal model for Parkinson's disease. MPTP injection led to an increase in the serum level of perforin in wild-type mice, but it is unlikely that this systemic increase is responsible for the deleterious local effects on the dopaminergic neurons in the substantia nigra. The fact that cytotoxic CD8+ T, NK and even CD4+ Th cells are the most prominent source of perforin [12, 13] combined with the findings that cytotoxic CD8+ T cells and CD4+ Th cells infiltrate brain parenchyma in PD animal models [7-9], make it very likely that these infiltrating cells are responsible for the perforin-mediated effects on the dopaminergic neurons. Moreover, it has been shown that perforin from NK cells, CD8+ T cells and CD4+ Th cells can mediate neurite damage [15-16] or neuron death [17] *in-vitro*. CD8+ T cells have been found in close proximity with activated microglia and degenerating neurons [28]. NK cells have been demonstrated capable to kill neurons through perforin-mediated cytotoxicity [29]. Interestingly, perforin may participate in PD development by inducing blood-brain barrier (BBB) disruption, as perforin has been shown to be an important component in inducing BBB disruption [21-22]; in this way, perforin may even contribute to an increase of the infiltration of deleterious lymphocytes.

As far as the acute decrease in striatal dopamine level is concerned: It has been demonstrated that lytic granules isolated from CD4+ T cells, CD8+ T cells and NK cells are able to induce axonal microtubule destabilization independent of apoptosis [16]. Microtubules provide platforms for intracellular transport and are involved in a variety of cellular processes, including the movement of secretory vesicles [27]. Perforin, as an important component of lytic granules, may participate in the granule-induced microtubule destabilization in SN DA neuron axons, impairing DA secretion in striatum as observed in the initial stage after MPTP-intoxication.

Four weeks post MPTP lesion, DA neurons recovered from the acute MPP+ shock and so the striatal dopamine level in both groups at this time point represents the capacity of surviving DA neurons to produce dopamine. The striatal dopamine level gradually recovered to 40% in wild type mice and to 80% in Pfp+/− mice. Accordingly, significant DA neuron loss in SN was found in wild-type mice (around 40%) 4 weeks post MPTP lesion; DA neuron loss was not significant in Pfp−/− mice. The attenuation of DA neuron loss by perforin-deficiency strongly demonstrates the involvement of perforin in this process.
It is evident that double immunostaining of the MPTP-lesioned mice brains for perforin and one of the specific lymphocyte markers can simply confirm the strong suggestion that these infiltrated cells are responsible for the perforin-mediated DA neuron damage. Practical problems, so far, have prevented us to successfully perform these experiments. Our present study, anyway, advocates for the development of a perforin-targeting therapy and the testing of currently existing anti-perforin antibodies [30-31] in our PD animal model.

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